Bs2 RESISTANCE GENE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. Application Serial No. 09/360,186, filed July, 23, 1999, which claims priority from U.S. Provisional Application No. 60/093,957, filed July 23, 1998; all of which are hereby incorporated herein in their entirety by reference.

FIELD OF THE INVENTION

This invention relates to plant disease resistance, in particular to plant genes conferring pathogen resistance.

BACKGROUND OF THE INVENTION

Plants are hosts to thousands of infectious diseases caused by a vast array of phytopathogenic fungi, bacteria, viruses, and nematodes. Plants recognize and resist many invading phytopathogens by inducing a rapid defense response, termed the hypersensitive response (HR). HR results in localized cell and tissue death at the site of infection, which constrains further spread of the infection. This local response often triggers non- specific resistance throughout the plant, a phenomenon known as systemic acquired resistance (SAR). Once triggered, SAR provides resistance for days to a wide

range of pathogens. The generation of the HR and SAR in a plant depends upon the interaction between a dominant or semi-dominant resistance (*R*) gene product in the plant and a corresponding dominant avirulence (*Avr*) gene product expressed by the invading phytopathogen. It has been proposed that phylopathogen Avr products function as ligands, and that plant R products function as receptors. Thus, in the widely held model of phytopathogen/plant interaction, binding of the Avr product of an invading pathogen to a corresponding R product in the plant initiates the chain of events within the plant that produces HR and SAR and ultimately leads to disease resistance.

The production of transgenic plants carrying a heterologous gene sequence is now routinely practiced by plant molecular biologists. Methods for incorporating an isolated gene sequence into an expression cassette, producing plant transformation vectors, and transforming many types of plants are well known. Examples of the production of transgenic plants having modified characteristics as a result of the introduction of a heterologous transgene include: U.S. Patent Nos. 5,719,046 to Guerineau (production of herbicide resistant plants by introduction of bacterial dihydropteroate synthase gene); 5,231,020 to Jorgensen (modification of flavenoids in plants); 5,583,021 to Dougherty (production of virus resistant plants); and 5,767,372 to De Greve and 5,500,365 to Fischoff (production of insect resistant plants by introducing *Bacillus thuringiensis* genes).

In conjunction with such techniques, the isolation of plant *R* genes has similarly permitted the production of plants having enhanced resistance to certain pathogens. Since the cloning of the first *R* gene, *Pto* from tomato, which confers resistance to *Pseudomonas syringae* pv. *tomato* (Martin *et al.*, 1993), a number of other *R* genes have been reported (Hammond-Kosack and Jones, 1997). A number of these genes have been used to introduce the encoded resistance characteristic into plant lines that were previously susceptible to the corresponding pathogen. For example, U.S. Patent No. 5,571,706 describes the introduction of the *N* gene into tobacco lines that are susceptible to Tobacco Mosaic Virus (TMV) in order to produce TMV-resistant tobacco plants. WO 95/28423 describes the creation of transgenic plants carrying the *Rps2* gene from *Arabidopsis thaliana*, as a means of creating resistance to bacterial pathogens including

Pseudomonas syringae, and WO 98/02545 describes the introduction of the *Prf* gene into plants to obtain broad-spectrum pathogen resistance.

Bacterial spot disease of tomato and pepper, caused by the phytopathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), can be devastating to commercial production of these crops in areas of the world with high humidity and heavy rainfall. While control of *Xcv* in commercial agriculture is based largely on the application of pesticides, genetic resistance to bacterial spot disease has been described in both tomato and pepper (Cook and Stall, 1963; Cook and Guevara, 1984; Kim and Hartmann, 1985; Jones and Scott, 1986). Of the two hosts, genetic resistance in pepper has been more well characterized. Several single loci (*Bs1*, *Bs2*, and *Bs3*) that confer resistance in a "gene-for-gene" manner have been identified (Hibberd *et al.*, 1987). Moreover, the corresponding avirulence genes (*avrBs1*, *avrBs2*, and *avrBs3*) have been cloned from *Xcv* (Swanson *et al.*, 1988; Minsavage *et al.*, 1990). Genetic and molecular characterization of these avirulence genes has provided a great deal of information concerning the interaction between *Xcv* and pepper (Kearney *et al.*, 1988; Kearney and Staskawicz, 1990; Herbers *et al.*, 1992; Van den Ackerveken *et al.*, 1996).

Of particular interest is the interaction governed by the avirulence gene *avrBs2* and the resistance gene *Bs2*. *AvrBs2* was originally identified as a 2.3 kb DNA fragment located on the *Xcv* chromosome (Minsavage *et al.*, 1990; Kearney, 1989). Recently, it was established that *avrBs2* encodes a protein with some homology to *A. tumefaciens* agrocinopine synthase and *E. coli* UgpQ, suggesting a possible enzymatic function (Swords *et al.*, 1996). Mutant *Xcv* strains in which the *avrBs2* gene has been disrupted or replaced are less virulent on susceptible hosts and are only able to grow to levels similar to that of wild type strains in a resistant host (Kearney, 1989; Kearney and Staskawicz, 1990; Swords *et al.*, 1996). In addition, a survey of various races of *Xcv* and other pathovars of *X. campestris* has shown that *avrBs2* is very widespread (Kearney and Staskawicz, 1990). For example, *avrBs2* activity was shown to be present in *Xc campestris* (the causative agent of black rot in crucifers), *Xc oryzae* (now termed *X. oryzae* pv. *oryzae* (the causative agent of bacterial blight in rice), *Xc citri* (now termed *X. axonopodis* (the causative agent of citrus canker) and *Xc phaseoli* (the causative agent of

bacterial blight of bean) (Kearney and Staskawicz, 1990). These studies also suggest that *avrBs2* plays a highly conserved role in the fitness of *X. campestris;* isolates having *avrBs2* show enhanced vigor on susceptible plant lines. The effectiveness of the *Bs2* resistance gene against the some of the major races of *Xcv* appears to be based on this dual phenotype (fitness and elicitation of *Bs2*-mediated HR response) of the *avrBs2* gene (Kearney and Staskawicz, 1990).

The availability of the *Bs2* gene would facilitate the production of transgenic plants having resistance to a potentially wide range of phylopathogens. It is to such a gene that the present invention is directed.

SUMMARY OF THE INVENTION

The invention provides an isolated *Bs2* gene from pepper that is shown to confer resistance to *Xanthomonas campestris* pv. *vesicatoria* when introduced into plants that are otherwise susceptible to infection by this organism. Specifically, such plants develop a hypersensitive response to the pathogen at the site of inoculation and show an enhanced resistance to systemic infection.

In one aspect of this invention, the nucleic acid sequences of the *Bs2* gene and cDNA from pepper are provided, as is the amino acid sequence of the pepper Bs2 protein. The functional hallmark of the Bs2 protein is that it has Bs2 biological activity: when co-expressed in a plant with a *Xanthomonas campestris AvrBs2* gene product, it produces a localized hypersensitive response, as determined by a transient assay technique described in detail below.

The existence of *Bs2* homologs in other plant species including tomato and tobacco is demonstrated, and the invention provides the structural features and functional characteristics of such homologs and teaches how they may be isolated. Because the pepper *Bs2* gene is the first *Bs2* gene to have been isolated, it is referred to as the prototypical *Bs2* gene; and the pepper Bs2 protein is referred to as the prototypical Bs2 protein. Homologs of the Bs2 protein are proteins that possess Bs2 biological activity and share a specified level of sequence identity with the prototype pepper Bs2 protein

(typically at least 50% amino acid sequence identity). Nucleic acid molecules that encode such homologs are also encompassed by the invention.

In another aspect of the invention, nucleic acid molecules are provided that comprise a minimum number of consecutive nucleotides of the disclosed *Bs2* sequences (e.g., at least 15 consecutive nucleotides of the pepper *Bs2* cDNA). These molecules are useful, among other things, as PCR primers for amplifying portions of a *Bs2* nucleic acid molecule, as sequencing primers to verify the authenticity of an amplified molecule, and as hybridization probes.

The invention also provides recombinant nucleic acid molecules that comprise a promoter sequence operably linked to a *Bs2* open reading frame. Such molecules may be introduced into plants so as to confer enhanced resistance to phytopathogens such as *Xanthomonas campestris*. Transgenic plants comprising these molecules are also provided by the invention. Plants that may be usefully transformed with a *Bs2* nucleic acid molecules to confer enhanced pathogen resistance include pepper, tomato, tobacco, broccoli, cauliflower, cabbage, cowpea, canola, bean, soybean, rice, corn, wheat, barley, citrus, cotton, cassava, grape and walnut.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic illustration of constructs introduced into plants in either the transient HR assay, or for stable transformation.

Sequence Listing

The nucleic and amino acid sequences listed in the accompanying sequence listing are showed using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

Seq. ID No. 1 shows the nucleic acid sequence of the pepper *Bs2* gene. The sequence comprises the following regions:

Nucleotides	Feature
1- 502	promoter region
503-554	exon 1 (5' untranslated region (UTR))
555-1439	intron 1
1440-1479	exon 2 (5' UTR (continued))
1480-4162	exon 2 continued (initiating ATG at 1480)
4163-31184	intron 2
31185-31216	exon 3
31217-31219	stop codon
31219-end	3' UTR and 3' regulatory region

Seq. ID No. 2 shows the nucleic acid sequence of the pepper *Bs2* cDNA.

Seq. ID No. 3 shows the amino acid sequence of the pepper Bs2 protein.

Seq. ID No. 4 shows the nucleic acid sequence of the pepper *Bs2* open reading frame (ORF).

Seq. ID Nos. 5-8 show primers that may be used to amplify certain portions of the pepper *Bs2* cDNA.

Seq. ID No. 9 shows the nucleic acid sequence of the pepper *Bs2* promoter.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the invention, the following definitions of terms are provided:

Bs2 protein biological activity: At a minimum, Bs2 protein biological activity refers to the ability of a protein to trigger HR when co-expressed in a plant with the gene product of *AvrBs2* as determined by the transient assay described below. Bs2 protein biological activity may also confer resistance to *Xanthomonas campestris* ("*Xc*") when expressed in a plant or a plant cell that would otherwise be susceptible to *Xc* infection. This resistance activity may readily be determined by challenging *Bs2*-expressing transgenic plants with *Xc*, as described below.

Bs2 protein: A protein having Bs2 protein biological activity and sharing amino acid sequence identity with the amino acid sequence of the prototypical Bs2 protein shown in Seq. ID No. 3 (the pepper Bs2 protein). Bs2 proteins that are more distantly related to the prototypical Bs2 protein will share at least 50% amino acid sequence identity with the sequence shown in Seq. ID No. 3, as determined by the methods described below. More closely related Bs2 proteins may share at least 60%, 65%, 70%, 75% or 80% sequence identity with the pepper Bs2 protein. Bs2 proteins that are most closely related to the pepper protein will have Bs2 protein biological activity and share at least 85%, 90% or 95% sequence identity with the pepper protein.

Bs2 gene / *Bs2* cDNA: Nucleic acid molecules that encode a Bs2 protein. Nucleic acid molecules that encode the pepper Bs2 protein are provided in Seq. ID No. 1 (pepper

Bs2 gene), Seq. ID No. 2 (pepper *Bs2* cDNA) and Seq. ID No. 4 (pepper *Bs2* ORF). The invention includes not only the nucleic acid molecules provided in Seq. ID Nos. 1, 2 and 4, but also homologs and orthologs of these sequences, nucleic acid molecules that encode Bs2 proteins, and probes and primers that are derived from these sequences.

Probes and primers: Nucleic acid probes and primers may readily be prepared based on the nucleic acids provided by this invention. A probe comprises an isolated nucleic acid attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Sambrook *et al.* (1989) and Ausubel *et al.* (1987).

Primers are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length. Primers may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in Sambrook *et al.* (1989), Ausubel *et al.* (1987), and Innis *et al.*, (1990). PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with its length. Thus, for example, a primer comprising 20 consecutive nucleotides of the pepper *Bs2* cDNA or gene will anneal to a target sequence such as a *Bs2* gene homolog from tomato contained within a tomato genomic DNA library with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers may be selected that comprise 20, 25, 30, 35, 40, 50 or more consecutive nucleotides of the pepper *Bs2* cDNA or gene sequences.

The invention thus includes isolated nucleic acid molecules that comprise specified lengths of the disclosed *Bs2* cDNA or gene sequences. Such molecules may comprise at least 20, 25, 30, 35, 40 or 50 consecutive nucleotides of these sequences and may be obtained from any region of the disclosed sequences. By way of example, the pepper *Bs2* cDNA and gene sequences may be apportioned into halves or quarters based on sequence length, and the isolated nucleic acid molecules may be derived from the first or second halves of the molecules, or any of the four quarters. The pepper *Bs2* cDNA, shown in Seq. ID No. 2 may be used to illustrate this. The pepper *Bs2* cDNA is 3099 nucleotides in length and so may be hypothetically divided into halves (nucleotides 1-1550 and 1551-3099) or quarters (nucleotides 1-775, 776-1550, 1551-2326 and 2327-3099). Nucleic acid molecules may be selected that comprise at least 20, 25, 30, 35, 40 or 50 consecutive nucleotides of any of these portions of the pepper cDNA. Thus, one such nucleic acid molecule might comprise at least 25 consecutive nucleotides of the region comprising nucleotides 1-1550 of the disclosed pepper cDNA.

Sequence identity: The similarity between two nucleic acid sequences, or two amino acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs of the pepper Bs2 protein will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman (1981); Needleman and Wunsch (1970); Pearson and Lipman (1988); Higgins and Sharp (1988); Higgins and Sharp (1989); Corpet *et al.* (1988); Huang el al. (1992); and Pearson *et al.* (1994). Altschul *et al.* (1994) presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MID) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. It can be accessed

at http://www.ncbi.nlm.nih.gov/BLAST/. A description of how to determine sequence identity using this program is available at http://www.ncbi.nlm.nih.gov/BLAST/blast help.html.

Orthologs of the disclosed pepper Bs2 protein are typically characterized by possession of at least 50% sequence identity counted over the full length alignment with the amino acid sequence of pepper Bs2 using the NCBI Blast 2.0, gapped blastp set to default parameters. Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 90% or at least 95% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85% or at least 90% or 95% depending on their similarity to the reference sequence. Methods for determining sequence identity over such short windows are described at http://www.ncbi.nlm.nih.gov/BLAST/blast FAQs.html. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided. The present invention provides not only the peptide homologs are described above, but also nucleic acid molecules that encode such homologs.

An alternative indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Conditions for nucleic acid hybridization and calculation of stringencies can be found in Sambrook *et al.* (1989) and Tijssen (1993). Nucleic acid molecules that hybridize under stringent conditions to the pepper *Bs2* sequences will typically hybridize to a probe based on either the entire pepper *Bs2* cDNA or selected portions of the cDNA under wash conditions of 0.2x SSC,

0.1% SDS at 65°C. A more detailed discussion of hybridization conditions is presented below.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequence that all encode substantially the same protein.

Specific binding agent: An agent that binds substantially only to a defined target. Thus a Bs2 protein specific binding agent binds substantially only the Bs2 protein. As used herein, the term "Bs2 protein specific binding agent" includes anti-Bs2 protein antibodies and other agents that bind substantially only to the Bs2 protein.

Anti-Bs2 protein antibodies may be produced using standard procedures described in a number of texts, including Harlow and Lane (1988). The determination that a particular agent binds substantially only to the Bs2 protein may readily be made by using or adapting routine procedures. One suitable in vitro assay makes use of the Western blotting procedure (described in many standard texts, including Harlow and Lane (1988)). Western blotting may be used to determine that a given Bs2 protein binding agent, such as an anti-Bs2 protein monoclonal antibody, binds substantially only to the Bs2 protein.

Oligonucleotide: A linear polynucleotide sequence of up to about 100 nucleotide bases in length.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

Transformed: A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with

plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Isolated: An "isolated" biological component (such as a nucleic acid or protein or organelle) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified Bs2 protein preparation is one in which the Bs2 protein is more enriched than the protein is in its natural environment within a cell. Generally, a preparation of Bs2 protein is purified such that the Bs2 represents at least 50% of the total protein content of the preparation. For particular applications, higher purity may be desired, such that preparations in which Bs2 represents at least 75% or at least 90% of the total protein content may be employed.

Ortholog: Two nucleotide or amino acid sequences are orthologs of each other if they share a common ancestral sequence and diverged when a species carrying that ancestral sequence split into two species. Orthologous sequences are also homologous sequences.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often

accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences that determine transcription. cDNA is synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into a peptide.

Transgenic plant: As used herein, this term refers to a plant that contains recombinant genetic material not normally found in plants of this type and which has been introduced into the plant in question (or into progenitors of the plant) by human manipulation. Thus, a plant that is grown from a plant cell into which recombinant DNA is introduced by transformation is a transgenic plant, as are all offspring of that plant that contain the introduced transgene (whether produced sexually or asexually).

II. Bs2 Protein and Nucleic acid Sequences

This invention provides Bs2 proteins and *Bs2* nucleic acid sequences, including cDNA and gene sequences. The prototypical *Bs2* sequences are the pepper sequences, and the invention provides for the use of these sequences to produce transgenic plants, such as pepper and tomato plants, having enhanced resistance to diseases cause by *Xanthomonas campestris*, such as bacterial spot disease. Because *Xc* causes disease in many plant species, and because the avirulence gene (*AvrBs2*) corresponding to *Bs2* is found in many *Xc pathovars*, *Bs2* will be useful to produce enhanced bacterial disease resistance in a wide variety of plant types.

A. Pepper *Bs2*

The pepper *Bs2* gene sequence is shown in Seq. ID No. 1. The sequence comprises 2 introns and 3 exons. Intron 1 is located within the 5' untranslated region of the gene, hence exon 1 contains only 5' untranslated sequence. Intron 2 is very large

(around 27 kb) and is located at the 3' end of the coding region. While the open reading frame continues across the 5' splice site of intron 2, resulting in a possible open reading frame encoding a hypothetical protein of 918 amino acids, no evidence has been found to suggest that this coding frame is actually utilized. Rather, only products in which intron 2 is spliced out are detected; splicing out intron 2 produces an open reading frame of 905 amino acids. This open reading frame is shown in Seq. ID No. 4, and the protein it encodes is shown in Seq. ID No. 3. A cDNA molecule corresponding to the spliced mRNA is shown in Seq. ID No. 2.

The pepper Bs2 protein includes a nucleotide binding motif and leucine rich repeats of the type that have been observed in other plant *R* genes (Leister *et al.*, 1996, Aarts *et al.*, 1998). As described in Examples 2 and 3 below, the pepper Bs2 protein has Bs2 biological activity, i.e., it mediates an *AvrBs2*-specific HR as determined by the *Agrobacterium* transient expression assay, and it produces a hypersensitive response following challenge of *Bs2*-expressing transgenic plants with *Xc*.

With the provision herein of the pepper *Bs2* cDNA and gene sequences, the polymerase chain reaction (PCR) may now be utilized in a preferred method for producing nucleic acid sequences encoding the pepper Bs2 protein. For example, PCR amplification of the pepper *Bs2* cDNA sequence may be accomplished either by direct PCR from a plant cDNA library or by Reverse-Transcription PCR (RT-PCR) using RNA extracted from plant cells as a template. *Bs2* gene sequences may be amplified from plant genomic libraries, or plant genomic DNA. Methods and conditions for both direct PCR and RT-PCR are known in the art and are described in Innis *et al.* (1990). Suitable plant libraries for direct PCR include the pepper YAC library described by Tai *et al.* (1995).

The selection of PCR primers will be made according to the portions of the cDNA (or gene) that are to be amplified. Primers may be chosen to amplify small segments of the cDNA, the open reading frame, the entire cDNA molecule or the entire gene sequence. Variations in amplification conditions may be required to accommodate primers of differing lengths; such considerations are well known in the art and are discussed in Innis *et al.* (1990), Sambrook *et al.* (1989), and Ausubel *et al* (1992). By

way of example only, the pepper *Bs2* cDNA molecule as shown in Seq. ID No. 2 (excluding the poly A tail) may be amplified using the following combination of primers:

primer 1 5' CAAATATTTCTTGGAGTGAATTTGA 3' (Seq. ID No. 5)

primer 2 5' AAAACTAAACTGGTTGTCTCATCGT 3' (Seq. ID No. 6)

The open reading frame portion of the cDNA may be amplified using the following primer pair:

primer 3 5' ATGGCTCATGCAAGTGTGGCTTCTC 3' (Seq. ID No. 7)

primer 4 5' CTAATGTTCTTCTGAATCAGAATCA 3' (Seq. ID No. 8)

These primers are illustrative only; it will be appreciated by one skilled in the art that many different primers may be derived from the provided cDNA and gene sequences in order to amplify particular regions of these molecules. Resequencing of PCR products obtained by these amplification procedures is recommended; this will facilitate confirmation of the amplified sequence and will also provide information on natural variation on this sequence in different ecotypes and plant populations. Oligonucleotides derived from the pepper sequence may be used in such sequencing methods.

Oligonucleotides that are derived from the pepper *Bs2* cDNA or gene sequences are encompassed within the scope of the present invention. Preferably, such oligonucleotide primers will comprise a sequence of at least 15-20 consecutive nucleotides of the pepper *Bs2* cDNA or gene sequences. To enhance amplification specificity, oligonucleotide primers comprising at least 25, 30, 35, 40, 45 or 50 consecutive nucleotides of these sequences may also be used.

B. Bs2 Genes in Other Plant Species

Orthologs of the *Bs2* gene are present in a number of plant species including tomato and tobacco (see Example 4 below). With the provision herein of the prototypical Bs2 protein from pepper and cDNA and gene sequences that encode this protein, the cloning by standard methods of cDNAs and genes that encode Bs2 protein orthologs in other plant species is now enabled. As described above, orthologs of the disclosed pepper Bs2 protein have Bs2 protein biological activity and are typically characterized by possession of at least 50% sequence identity counted over the full length alignment with

the amino acid sequence of pepper Bs2 using the NCBI Blast 2.0, gapped blastp set to default parameters. Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 90% or at least 95% sequence identity.

Both conventional hybridization and PCR amplification procedures may be utilized to clone sequences encoding Bs2 protein orthologs. Common to both of these techniques is the hybridization of probes or primers derive from the pepper *Bs2* cDNA or gene sequence to a target nucleotide preparation, which may be, in the case of conventional hybridization approaches, a cDNA or genomic library or, in the case of PCR amplification, a cDNA or genomic library, or an mRNA preparation.

Direct PCR amplification may be performed on cDNA or genomic libraries prepared from the plant species in question, or RT-PCR may be performed using mRNA extracted from the plant cells using standard methods. PCR primers will comprise at least 15 consecutive nucleotides of the pepper *Bs2* cDNA or gene. One of skill in the art will appreciate that sequence differences between the pepper *Bs2* cDNA or gene and the target nucleic acid to be amplified may result in lower amplification efficiencies. To compensate for this, longer PCR primers or lower annealing temperatures may be used during the amplification cycle. Where lower annealing temperatures are used, sequential rounds of amplification using nested primer pairs may be necessary to enhance specificity.

For conventional hybridization techniques the hybridization probe is preferably conjugated with a detectable label such as a radioactive label, and the probe is preferably of at least 20 nucleotides in length. As is well known in the art, increasing the length of hybridization probes tends to give enhanced specificity. The labeled probe derived from the pepper cDNA or gene sequence may be hybridized to a plant cDNA or genomic library and the hybridization signal detected using means known in the art. The hybridizing colony or plaque (depending on the type of library used) is then purified and the cloned sequence contained in that colony or plaque isolated and characterized.

Orthologs of the pepper Bs2 may alternatively be obtained by immunoscreening of an expression library. With the provision herein of the disclosed pepper *Bs2* nucleic acid sequences, the enzyme may be expressed and purified in a heterologous expression system (e.g., *E. coli*) and used to , raise antibodies (monoclonal or polyclonal) specific for the pepper Bs2 protein. Antibodies may also be raised against synthetic peptides derived from the pepper Bs2 amino acid sequence presented herein. Methods of raising antibodies are well known in the art and are described in Harlow and Lane (1988). Such antibodies can then be used to screen an expression cDNA library produced from the plant from which it is desired to clone the *Bs2* ortholog, using routine methods. The selected cDNAs can be confirmed by sequencing and enzyme activity.

C. Bs2 Sequence Variants

With the provision of the pepper Bs2 protein and *Bs2* cDNA and gene sequences herein, the creation of variants of these sequences is now enabled.

Variant Bs2 proteins include proteins that differ in amino acid sequence from the pepper Bs2 sequence disclosed but which retain Bs2 protein biological activity. Such proteins may be produced by manipulating the nucleotide sequence of the pepper *Bs2* cDNA or gene using standard procedures such as site-directed mutagenesis or the polymerase chain reaction. The simplest modifications involve the substitution of one or more amino acids for amino acids having similar biochemical properties. These so-called conservative substitutions are likely to have minimal impact on the activity of the resultant protein. Table 1 shows amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions.

TABLE 1

Original Residue	Conservative Substitutions
Ala	ser
Arg	lys
Asn	gln, his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu, val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

More substantial changes in enzymatic function or other features may be obtained by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions which in general are expected to produce the

greatest changes in protein properties will be those in which (a) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine. The effects of these amino acid substitutions or deletions or additions may be assessed for Bs2 protein derivatives by analyzing the ability of the derivative proteins to confer *Xc* resistance in the assays described below.

Variant *Bs2* cDNA or genes may be produced by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook *et al.* (1989), Ch. 15. By the use of such techniques, variants may be created which differ in minor ways, from the pepper *Bs2* cDNA or gene sequences disclosed, yet which still encode a protein having Bs2 protein biological activity. DNA molecules and nucleotide sequences which are derivatives of those specifically disclosed herein and which differ from those disclosed by the deletion, addition or substitution of nucleotides while still encoding a protein that has Bs2 protein biological activity are comprehended by this invention. In their simplest form, such variants may differ from the disclosed sequences by alteration of the coding region to fit the codon usage bias of the particular organism into which the molecule is to be introduced.

Alternatively, the coding region may be altered by taking advantage of the degeneracy of the genetic code to alter the coding sequence in such a way that, while the nucleotide sequence is substantially altered, it nevertheless encodes a protein having an amino acid sequence identical or substantially similar to the disclosed pepper Bs2 protein sequence. For example, the second amino acid residue of the pepper Bs2 protein is alanine. This is encoded in the pepper *Bs2* open reading frame (ORF) by the nucleotide codon triplet GCT. Because of the degeneracy of the genetic code, three other nucleoticle codon triplets—GCA, GCC and GCG—also code for alanine. Thus, the nucleotide sequence of the pepper *Bs2* ORF could be changed at this position to any of these three codons without affecting the amino acid composition of the encoded protein

or the characteristics of the protein. Based upon the degeneracy of the genetic code, variant DNA molecules may be derived from the cDNA and gene sequences disclosed herein using standard DNA mutagenesis techniques as described above, or by synthesis of DNA sequences. Thus, this invention also encompasses nucleic acid sequences which encode a Bs2 protein but which vary from the disclosed nucleic acid sequences by virtue of the degeneracy of the genetic code.

Variants of the Bs2 protein may also be defined in terms of their sequence identity with the prototype Bs2 protein shown in Seq. ID No. 4. As described above, Bs2 proteins have Bs2 biological activity and share at least 60% sequence identity with the pepper Bs2 protein. Nucleic acid sequences that encode such proteins may readily be determined simply by applying the genetic code to the amino acid sequence of a Bs2 protein, and such nucleic acid molecules may readily be produced by assembling oligonucleotides corresponding to portions of the sequence.

Nucleic acid molecules which are derived from the pepper *Bs2* cDNA and gene sequences disclosed include molecules that hybridize under stringent conditions to the disclosed prototypical *Bs2* nucleic acid molecules, or fragments thereof. Stringent hybridization conditions are hybridization at 65°C in 6 x SSC, 5 x Denhardt's solution, 0.5% SDS and 100 μg sheared salmon testes DNA, followed by 15-30 minute sequential washes at 65°C in 2 x SSC, 0.1% SDS, followed by 1 x SSC, 0.1% SDS and finally 0.2 x SSC, 0.1% SDS.

Low stringency hybridization conditions (to detect less closely related homologs) are performed as described above but at 50°C (both hybridization and wash conditions); however, depending on the strength of the detected signal, the wash steps may be terminated after the first 2 x SSC, 0.1% SDS wash.

The pepper *Bs2* gene or cDNA, and orthologs of these sequences from other plants, may be incorporated into transformation vectors and introduced into plants to produce enhanced disease resistance in such plants, as described in Example three below.

III. Introducing *Bs2* into Plants

Once a cDNA (or gene) encoding a protein involved in the determination of a particular plant characteristic has been isolated, standard techniques may be used to express the cDNA in transgenic plants in order to modify that particular plant characteristic. The basic approach is to clone the cDNA into a transformation vector, such that it is operably linked to control sequences (e.g., a promoter) that direct expression of the cDNA in plant cells. The transformation vector is then introduced into plant cells by one of a number of techniques (e.g., electroporation) and progeny plants containing the introduced cDNA are selected. Preferably all or part of the transformation vector will stably integrate into the genome of the plant cell. That part of the transformation vector which integrates into the plant cell and which contains the introduced cDNA and associated sequences for controlling expression (the introduced "transgene") may be referred to as the recombinant expression cassette.

Selection of progeny plants containing the introduced transgene may be made based upon the detection of an altered phenotype. Such a phenotype may result directly from the cDNA cloned into the transformation vector or may be manifested as enhanced resistance to a chemical agent (such as an antibiotic) as a result of the inclusion of a dominant selectable marker gene incorporated into the transformation vector.

Successful examples of the modification of plant characteristics by transformation with cloned cDNA sequences are replete in the technical and scientific literature. Selected examples, which serve to illustrate the knowledge in this field of technology, include:

U.S. Patent No. 5,571,706 ("Plant Virus Resistance Gene and Methods");

U.S. Patent No. 5,677,175 ("Plant Pathogen Induced Proteins");

U.S. Patent No. 5,510,471 ("Chimeric Gene for the Transformation of Plants");

U.S. Patent No. 5,750,386 ("Pathogen-Resistant Transgenic Plants");

U.S. Patent No. 5,597,945 ("Plants Genetically Enhanced for Disease Resistance");

U.S. Patent No. 5,589,615 ("Process for the Production of Transgenic Plants with Increased Nutritional Value Via the Expression of Modified 2S Storage Albumins");

U.S. Patent No. 5,750,871 ("Transformation and Foreign Gene Expression in Brassica Species");

U.S. Patent No. 5,268,526 ("Overexpression of Phytochrome in Transgenic Plants");

U.S. Patent No. 5,262,316 ("Genetically Transformed Pepper Plants and Methods for their Production"); and

U.S. Patent No. 5,569,831 ("Transgenic Tomato Plants with Altered Polygalacturonase Isoforms").

These examples include descriptions of transformation vector selection, transformation techniques and the construction of constructs designed to over-express the introduced cDNA. In light of the foregoing and the provision herein of the *Bs2* cDNA and gene sequences, it is thus apparent that one of skill in the art will be able to introduce these nucleic acids, or homologous or derivative forms of these molecules, into plants in order to produce plants having enhanced Bs2 activity. Expression of *Bs2* in plants that are otherwise sensitive to certain bacterial diseases, such as bacterial spot diseases, will be useful to confer enhanced resistance to these diseases. Expression of a *Bs2* transgene in plants that already express Bs2 protein may be used to further boost pathogen resistance.

A. Plant Types

Bacterial diseases affect many plant species, and in particular, *Xanthomonas campestris* can infect a wide variety of plants. At the molecular level, not only are orthologs of *Bs2* found in a number of plant species, but the *avrBs2* gene is found in a wide variety of *Xc* pathovars (Kearney & Staskawicz, 1990) that cause disease in many different plants including tomato, pepper, rice, citrus, cowpea, walnut, brassica (broccoli, cauliflower, cabbage), soybean, bean, alfalfa and grapes. Thus, the Bs2 protein may be usefully expressed in a wide range of higher plants to confer enhanced resistance to

bacterial disease, both monocotyledonous and dicotyledenous plants, including, but not limited to maize, wheat, rice, barley, soybean, cotton, beans in general, rape/canola, alfalfa, flax, sunflower, safflower, brassica, cotton, tobacco, flax, peanut, clover, cowpea, grapes; vegetables such as lettuce, tomato, cucurbits, cassava, potato, carrot, radish, pea, lentils, cabbage, cauliflower, broccoli, Brussels sprouts, peppers; tree fruits such as citrus, apples, pears, peaches, apricots, walnuts; and flowers such as carnations and roses.

B. Vector Construction, Choice of Promoters

A number of recombinant vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described including those described in Pouwels *et al.*, (1987), Weissbach and Weissbach, (1989), and Gelvin *et al.*, (1990). Typically, plant transformation vectors include one or more cloned plant genes (or cDNAs) under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant transformation vectors typically also contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Examples of constitutive plant promoters that may be useful for expressing the cDNA include: the cauliflower mosaic virus (CaMV) 35S promoter, which confers constitutive, high-level expression in most plant tissues (*see, e.g.*, Odel *et al.*, 1985, Dekeyser *et al.*, 1990, Terada and Shimamoto, 1990; Benfey and Chua. 1990); the nopaline synthase promoter (An *et al.*, 1988); and the octopine synthase promoter (Fromm *et al.*, 1989).

A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals, also can be used for expression of the cDNA in plant cells, including promoters regulated by: (a) heat (Callis *et al.*, 1988;. Ainley, *et al.* 1993; Gilmartin *et al.* 1992); (b) light (*e.g.*, the pea rbcS-3A promoter, Kuhlemeier *et al.*, 1989, and the maize rbcS promoter, Schaffner and Sheen, 1991); (c) hormones, such as abscisic acid (Marcotte *et al.*, 1989); (d) wounding (*e.g., wunl*,

Siebertz *et al.*, 1989); and (e) chemicals such as methyl jasminate or salicylic acid (*see also* Gatz *et al.*, 1997).

Alternatively, tissue specific (root, leaf, flower, and seed for example) promoters (Carpenter *et al.* 1992, Denis *et al.* 1993, Opperman *et al.* 1993, Stockhause *et al.* 1997; Roshal *et al.*, 1987; Schernthaner *et al.*, 1988; and Bustos *et al.*, 1989) can be fused to the coding sequence to obtained particular expression in respective organs.

Alternatively, the native *Bs2* gene promoter may be utilized. The *Bs2* gene promoter is contained within the ca. 2 kb sequence shown in Seq. ID No. 9. The 3' end of this sequence ends at the transcriptional start site for the *Bs2* gene (equivalent to nucleoticle number 503 in Seq. ID No. 1). One of skill in the art will appreciate that less than this entire sequence may be used in order to obtain effective promoter activity. The determination of whether a particular region of this sequence confers effective promoter activity may readily be ascertained by operably linking the selected sequence region to the *Bs2* cDNA (in conjunction with suitable 3' regulatory region, such as the NOS 3' regulatory region as discussed below) and determining whether the resulting construct is able to elicit HR response in the transient assay described below. Promoter regions that comprise, for example, nucleotides 250-2053, 500-2053, 1000-2053 or 1500-2053 of Seq. ID No. 9 may readily be employed.

Plant transformation vectors may also include RNA processing signals, for example, introns, which may be positioned upstream or downstream of the ORF sequence in the transgene. In addition, the expression vectors may also include additional regulatory sequences from the 3'-untranslated region of plant genes, *e.g.*, a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase (NOS) 3' terminator regions. The *Bs2* gene 3' regulatory sequence may also be employed.

Finally, as noted above, plant transformation vectors may also include dominant selectable marker genes to allow for the ready selection of transformants. Such genes include those encoding antibiotic resistance genes (e.g., resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin) and herbicide resistance genes (e.g., phosphinothricin acetyltransferase).

C. Arrangement of *Bs2* Sequence in Vector

The particular arrangement of the *Bs2* sequence in the transformation vector will be selected according to the type of expression of the sequence that is desired.

In most instances, enhanced Bs2 activity is desired, and the *Bs2* ORF is operably linked to a constitutive high-level promoter such as the CaMV 35S promoter. As noted above, enhanced Bs2 activity may also be achieved by introducing into a plant a transformation vector containing a variant form of the *Bs2* cDNA or gene, for example a form which varies from the exact nucleotide sequence of the *Bs2* ORF, but which encodes a protein that retains *Bs2* biological activity.

D. Transformation and Regeneration Techniques

Transformation and regeneration of both monocotyledonous and dicotyledonous plant cells is now routine, and the appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant to be transformed; those skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene glycol (PEG) mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and *Agrobacterium tumefaciens* (AT) mediated transformation. Typical procedures for transforming and regenerating plants are described in the patent documents listed at the beginning of this section.

E. Selection of Transformed Plants

Following transformation and regeneration of plants with the transformation vector, transformed plants are usually selected using a dominant selectable marker incorporated into the transformation vector. Typically, such a marker will confer antibiotic resistance on the seedlings of transformed plants, and selection of

transformants can be accomplished by exposing the seedlings to appropriate concentrations of the antibiotic.

After transformed plants are selected and grown to maturity, they can be assayed using the methods described herein to determine whether the susceptibility of the plant to *Xc* infection has been altered as a result of the introduced transgene.

IV. Production of Recombinant Bs2 Protein in Heterologous Expression Systems

Many different expression systems are available for expressing cloned nucleic acid molecules. Examples of prokaryotic and eukaryotic expression systems that are routinely used in laboratories are described in Chapters 16-17 of Sambrook *et al.* (1989). Such systems may be used to express Bs2 at high levels to facilitate purification of the protein. The purified Bs2 protein may be used for a variety of purposes. For example, the purified recombinant enzyme may be used as an immunogen to raise anti-Bs2 antibodies. Such antibodies are useful as both research reagents (such as in the study of phytopathogen defense mechanisms in plants) as well as diagnostically to determine expression levels of the protein in plants that are being developed for agricultural use. Thus, the antibodies may be used to quantify the level of Bs2 protein both in existing plant varieties and in transgenic varieties that are designed to over-express the Bs2 protein. Such quantification may be performed using standard immunoassay techniques, such as ELISA and in situ immunofluorescence and others described in Harlow & Lane (1988).

By way of example only, high level expression of the Bs2 protein may be achieved by cloning and expressing the *Bs2* cDNA in yeast cells using the pYES2 yeast expression vector (Invitrogen, Carlsbad, CA). Alternatively, a genetic construct may be produced to direct secretion of the recombinant Bs2 protein from the yeast cells into the medium. This approach will facilitate the purification of the Bs2 protein, if this is necessary. Secretion of the recombinant protein from the yeast cells may be achieved by placing a yeast signal sequence adjacent to the *Bs2* coding region. A number of yeast signal sequences have been characterized, including the signal sequence for yeast invertase. This sequence has been successfully used to direct the secretion of

heterologous proteins from yeast cells, including such proteins as human interferon (Chang *et al.*, 1986), human lactoferrin (Liang and Richardson, 1993) and prochymosin (Smith *et al.*, 1985).

Alternatively, the enzyme may be expressed at high level in prokaryotic expression systems, such as *E. coli*, as described in Sambrook *et al.* (1989). Commercially available prokaryolic expression systems include the pBAD expression system and the ThioFusion expression system (Invitrogen, Carlsbad, CA).

EXAMPLES

Example 1: Cloning Pepper *Bs2*

The *Bs2* gene was isolated by positional cloning. Molecular markers tightly linked to the *Bs2* gene were identified by randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analysis and a high resolution genetic map of the locus was constructed. The closest markers were used to screen a yeast artificial chromosome (YAC) library of a pepper cultivar containing the *Bs2* gene and two clones spanning the locus were identified (i.e. chromosome landing). Further high resolution mapping facilitated the physical delimitation of the locus to a region of approximately 103 kb which was completely sequenced. The *Bs2* gene was identified by testing candidates (of which there were only two) using the *Agrobacterium*-mediated transient assay described below.

Example 2: Transient Expression Assay for *Bs2*

To test *Bs2* candidates for functional activity, an *Agrobacterium*-mediated transient transformation assay was used. Constructs for expression in the *Agrobacterium*-mediated transient assay were made using the pMD1 binary expression vector. The pMD1 vector is a derivative of pBI121 (Clontech, Palo Alto, CA) in which the GUS

reporter gene has been replaced with a synthetic polylinker (M. Dixon, unpublished). The pDD5 construct consists of the *avrBs2* orf (Swords *et al.* 1996) cloned between the 35S promoter and the NOS terminator sequences of pMD1 . This construct was mobilized into *A. tumefaciens* strain C58C1 (pCH32) using standard methods. The pCH32 plasmid contains the VirE and VirG genes (A. Hamilton, unpublished) and was constructed by cloning the VirE operon from pSW108 (Winans *et al.*, 1987) into the Pvull site of pCH30 (a derivative of the pCC 113 (Chen *et al.*, 1991). Cells containing the construct were grown in a 5ml L-broth culture containing antibiotics (tetracycline 5 μg/ml and kanamycin 50 μg/ml) overnight. This culture was used inoculate a 50 ml L-broth culture containing antibiotics (tetracycline 5 ug/mI and kanamycin 50 μg/ml), 10 mM MES, and 20 μM acetosyringone. Following overnight growth, bacteria were collected by centrifugation and resuspended in 10mM MgCl2, 10 mM MES, and 150 μM acetosyringone to a final OD_{600} Of 0.6. After 2-3 hours, about 10 μl of the cells were hand-infiltrated into intercellular leaf spaces of pepper cultivars with and without the *Bs2* resistance gene using a plastic transfer pipet (a modification of the method described by Minsavage *et al.*, 1990). After 24 hours, a characteristic macroscopic hypersensitive response comprising browning of tissue and necrosis in the infiltrated area (HR, Minsavage *et al.* 1990) was observed only in pepper plants containing the *Bs2* resistance gene. No macroscopic reaction was observed in the pepper plants lacking the *Bs2* gene. These results show that the transient assay system is able to elicit a detectable HR response when the *Bs2* and avrBs2 genes are simultaneously expressed in the same cells of the pepper plant.

A variation on this assay was then utilized to asses the ability of various forms of the *Bs2* gene to trigger HR response. This assay is essentially performed by co-infiltrating leaves of susceptible plants with two *Agrobacterium* clones, one containing *avrBs2*, the other containing the *Bs2* construct. HR response is typically manifested within 48 hours as browning and necrosis within the area of infiltration.

Four constructs comprising various forms of the *Bs2* gene were produced. The constructs, as depicted in Fig. 1 comprise: the *Bs2* cDNA operably linked to 35S promoter and the NOS 3' regulatory region (construct X5); the same construct having

intron 1 inserted at the position that the intron occurs in the *Bs2* gene (construct XO5); a construct that is identical to construct XO5 except that the 35S promoter is replaced with the native *Bs2* promoter (shown in Seq. ID No. 9) (construct E.4). Construct E.4 + 2 comprises the *Bs2* gene (including the *Bs2* promoter and 3' regulatory regions) with a truncated form of intron 2 of *Bs2*.

To make these transient expression constructs using the *Bs2* gene, adapter primers containing an XbaI site were designed for PCR amplification of the 5' end of the *Bs2* gene. For the X5 construct, the primer was 5' CCTCTAGA**ATGGCTCATGCAAGTGTGCGTTCTCTTATG** 3' (underlined sequence is the XbaI site, bolded sequence encodes the first 10 amino acids of Bs2). For the XO5 construct which includes the first intron located in the 5' UTR sequence, the primer was 5'CCTCTAGA**C**AAAATATTTCTTGGAGTGAATTTGA 3' (underlined sequence is the XbaIsite, bolded letter is the transcriptional start site of *Bs2*). For both constructs the second primer used for amplification was 5' CCATCCCACACTTCACAACTCCA 3'. Amplified products were cloned and sequenced to check fidelity of the clones. Clones for both constructs were digested with XbaI and SalI and ligated to pMD1 vector that had been digested with XbaI and SalI. The majority of the *Bs2* gene was isolated as a SalI-EcoRI fragment from a cosmid that was cloned into pBluescript KS + (Stratagene, La Jolla, CA). The 3' ends of the two constructs were derived from PCR amplification of the appropriate 3' RACE product using the primers 5' GTCCTTGAGCGCCTCATG 3'and 5' ACTAAACTGGGTGTCTCATCGT 3'. This PCR products was cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced to check the fidelity of the clone. The 3' end fragment was isolated by digesting the pCRII-TOPO clone with EcoRI and ligating the fragment to the SalI-EcoRI pBluescript KS + construct that had been digested with EcoRI. Proper orientation of the EcoRI 3' end fragment was determined by sequencing. This construct was digested with SalI and SacI (the SacIsite is in the plasmid polylinker) and ligated to the initial pMD1 constructs which had been digested with SalI and SacI, to produce the X5 and XO5 constructs.

Transient assay constructs (E.4 and E.4 + 2) to examine the native promoter region of the *Bs2* were made by digesting a clone of the cosmid contig spanning the *Bs2*

locus with DraIII which cuts approximately 2050 bp upstream of the transcriptional start site. Following digestion, T4 DNA polymerase was used to fill in the DraIII end and digestion with EcoRI was performed. This resulted in the liberation of a fragment of approximately 5.6 kb (from the DraIII site to the EcoRI site located at position 2620 of the cDNA). This fragment was cloned into the binary cosmid vector pCLD04541 (Bent *et al.*, 1994) which had been previously digested with XhoI, subjected to T4 DNA polymerase treatment and then digested with EcoRI. Ligation of the 5.6 kb fragment into the vector resulted in the elimination of the DraIII and XhoI sites. For construct E.4, the 3' end of *Bs2* was isolated from the pCRII-TOPO clone by digestion with EcoRI as described for the construction of the X5 and XO5 constructs. This clone was ligated to the 5.6 kb pCLD04541 construct at the EcoRI site to yield the E.4 construct. Clones were sequenced to ensure proper orientation of the EcoRI fragment.

To obtain the E.4 + 2 construct, the E.4 construct was digested with XhoI (XhoI site al position 1784 of the cDNA, position 3263 of the genomic sequence) and SpeI (which cuts at the SpeI site in the vector polylinker) and this region was replaced with a XhoI-SpeI (position 3263 to 7539 of the *Bs2* genomic sequence) fragment of about 4.3 kb isolated from a *Bs2* cosmid clone. This intermediate construct was digested with XbaI which cuts a site about 2.5 kb past the start of intron 2 (position 6648 of the *Bs2* genomic sequence) and a site in the pCLD04541 vector. The smaller XbaI fragment (containing 1.1 kb of intron 2 from position 6648 to 7539) was replaced with an XbaI fragment of about 3 kb containing part of the 3' end of intron 2 (about 1.2 kb) and the 3' end of the NBS-LRR candidate and regulatory elements located about 1.7 kb downstream of the end of the orf (XbaI site at position 29957 in *Bs2* genomic sequence, end of intron 2 at position 31184, end of orf 31216).

Strains containing these constructs were grown as described above for the 35S-*avrBs2* construct. Following resuspension in the final buffer, a volume of cells containing the candidate constructs were mixed with an equal volume of cells containing the 35S-*avrBs2* construct. The resulting effective concentration of the cells was OD_{600} = 0.3. These cells were then hand-infiltrated into the intercellular leaf space as described above. As controls, suspensions of cells with the 35S-*avrBs2* construct only and

suspensions of cells with the *Bs2* constructs only were infiltrated in comparable areas of the same leaves. Three different plant species were used in this assay: susceptible pepper cultivar ECW (i.e. *bs2/bs2*), susceptible tomato cultivar Walter, and non-host *Nicotiana benthamiana*. HR responses were typically observed within 48 hours and varied depending on the construct and the host plant. The results are shown in Table 2 below.

TABLE 2

Agrobacterium-mediated transient transformation assay

Construct	N. benthamiana	Pepper (ECW)	Tomato (Walter)
35S-avrBs2	-	-	-
X5	-	-	-
XO5	-	-	-
E.4	-	-	-
E.4 + 2	-	-	-
X5/35S-avrBs2	++++	++	+
XO5/35S-avrBs2	++	+	-
E.4/35S-avrBs2	+/-	-	-
E.4 + 2/35S-avrBs2	+/-	-	-

Reactions were scored at 48 hours post-infiltration. Plants were grown under greenhouse conditions. Following infiltration, plants were placed in growth chamber under standard conditions during the length of the assay.

Scoring scale + + + + strong HR characterized by confluent necrosis of the area infiltrated/ strong browning of collapsed tissue (typical of *Xcv avrBs2lBs2* pepper reaction); + + moderate HR characterized by necrosis over the entire infiltrated area, but not complete collapse of the area, tissue clearly showing browning; + weak HR some collapse in the area of infiltration, light browning of the area; - no HR, some wound associated browning localized to point of infiltration, but not to entire infiltrated area).

These results indicate that the introduction of *Bs2* constructs into various species of susceptible plants is sufficient to trigger HR in the presence of *AvrBs2*.

Example 3: Stable Transformation of *Bs2* into Plants

The X5, XO5, E.4 and E.4 + 2 constructs were stably transformed into pepper, tomato and tobacco varieties described above using standard procedures.

Analysis of the *Xc* resistance phenotypes of these transgenic plants is determined by challenging plants with *Xcv* by hand infiltration as described above for the *Agrobacterium* transient expression assay. HR is determined by visual inspection.

Example 4: *Bs2* Homologs

As noted above, homologs of *Bs2* exist in a number of plant species including pepper, tomato and tobacco. The existence of these sequences may be demonstrated by hybridization techniques, such as Southern blotting. Southern blotting using high stringency hybridization conditions reveals the presence of *Bs2* homologs in pepper and tomato. Hybridization was performed using probes based on different portions of the *Bs2* gene sequence. Probes were hybridized to tomato genomic DNA, and to genomic DNA from *Xc* pv. *vesicatoria* resistant and susceptible lines of *Capsicum annuum*. Hybridization was performed at 65°C in 6 x SSC, 5 x Denhardt's solution, 0.5 % SDS and 100 μg sheared salmon testes DNA, followed by 15-30 minute sequential washes at 65°C in 2 x SSC, 0.1 % SDS, followed by 1 x SSC, 0.1% SDS and finally 0.2 x SSC, 0.1% SDS.

Under these conditions, a probe comprising nucleotides 1-927 of Seq. ID No. 1 (and 107 nucleotides 5' of this sequence, shown in Seq. ID No. 9) shows hybridization to a single band in the *Xcv* resistant pepper, and no hybridization to the *Xcv* susceptible pepper. A probe comprising nucleoticles 1042-2239 of Seq. ID No. 1 showed hybridization to approximately 15 bands in both resistant and susceptible pepper, and to a single band in tomato.

Lower stringency hybridization conditions are used to detect *Bs2* homologs in less closely related species. For example, hybridization of either of these probes under low stringency hybridization conditions as described above is used to detect homologs from brassica and other plant species. Once a *Bs2*-hybridizing band is detected in a plant species, standard techniques such as screening cDNA or genomic libraries from the plant with the *Bs2* probe may be used. Alternatively, *Bs2* homologs may be isolated by screening an expression library from the plant in question using a Bs2 protein specific binding agent, such as an anti-Bs2 antibody produced as described above. Such homologs may be introduced into plants using the methods described above in order to produce enhanced resistance to *Xc* pathogens.

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